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Polymyxin B, a novel inhibitor of red cell Ca^{2+}-activated K^+ channel

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Polymyxin B (PXB), a cyclic peptide antibiotic, in concentrations 0.1–3.0 mg/ml (0.08–4.0 mmol/l), inhibited the K^+ efflux induced by opening of the Ca^{2+}-activated K^+ channel (the Gárdos effect) in intact human red blood cells. The inhibition was observed when the Gárdos effect was elicited by Ca^{2+} in the presence of vanadate, or propranolol, in ATP-depleted cells, and in A23187-treated cells. The inhibition of the Gárdos effect is caused neither by the inhibition of the anion channel by PXB nor by the inhibition of Ca^{2+} entry. It can be ascribed to the inhibition of the Ca^{2+}-activated K^+ channel. The mechanism of the inhibition remains to be elucidated.

Ca^{2+}-activated K^+ channel; Polymyxin B; Ion channel inhibition; (Human red cell)

1. INTRODUCTION

The opening of the Ca^{2+}-activated K^+ channel first described by Gárdos [1] in human red cells has been established during the last three decades as a ubiquitous cell response to the increased cytoplasmic Ca^{2+} concentration (reviews [2,3]). In excitable cells it may play a role in the repolarization of the membrane [4]. Its function in non-excitable cells is unknown. Several authors [5,6] suggested that its activity could participate in the modulation of the Ca^{2+} influx. It is feasible that inhibitors of the Ca^{2+}-activated K^+ channel could be useful in corroborating such suggestions. In fact, it has been shown that quinidine, a well known inhibitor of the Ca^{2+}-activated K^+ channel [7], inhibits the $^{45}Ca^{2+}$ uptake in vanadate-treated red cells [6]. Furthermore, a parallel inhibition of both $^{45}Ca^{2+}$ uptake and the Gárdos effect by divalent cations and HS-reagents was observed [8].

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Here we present results which show that polymyxin B (PXB), an inhibitor of protein kinase C [9], inhibits the Ca^{2+}-activated K^+ channel in human red cells without inhibiting the $^{45}Ca^{2+}$ uptake.

2. EXPERIMENTAL

Chelatonate-treated blood was used in experiments as described [8]. The measurement of the Gárdos effect and the $^{45}Ca^{2+}$ uptake in vanadate-treated red cells were also described in [8]. The propranolol-induced Gárdos effect [10] was studied as the vanadate-induced one except 0.5 mM propranolol was used instead of vanadate. Red cells were depleted of ATP by a 5 h preincubation at 37°C with 12.5 mM inosine and 5 mM iodoacetamide, and then treated as described for vanadate-treated cells. The membrane potential changes were measured as described by Sims et al. [11] using the fluorescence probe 3,3′-dipropylthiodicarbocyanine iodide (a kind gift of Dr A. Waggoner of Amherst College, Amherst, MA, USA). The incorporation of $^{32}P_i$ into membrane

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proteins was performed as follows: 1.2 ml of a 30% suspension was preincubated with 18.5 MBq ^{32}P-labelled orthophosphate (spec. act. 370 MBq/mg P_i) for 1 h at 25°C. 0.1-ml aliquots were incubated with tested compounds for 30 min at 25°C. The incubation was stopped by the addition of 1 ml of ice-cold 0.3 M glycerol followed by a rapid centrifugation in a microcentrifuge. Supernatants were carefully aspirated and pellets were dissolved in the sample buffer for SDS-polyacrylamide gel electrophoresis. The latter was performed according to Laemmli [12] using 10% gel. The gels were silver-stained and cellophane-dried. The label was detected by autoradiography. PXB was obtained from Galena, Opava, Czechoslovakia, courtesy of Dr Čech.

3. RESULTS

We studied the effect of PXB on the Gárdos effect induced by vanadate. PXB inhibited the Gárdos effect in a concentration-dependent manner, in the concentration range of 0.1–5.0 mg/ml (0.08–4.0 mmol/l) (fig.1A). The inhibitory effect of PXB is not caused by its interference with vanadate-linked triggering mechanisms [6,13]. This is substantiated by the fact that the Gárdos effect induced by other, chemically quite different, reagents is also inhibited by PXB. Thus, PXB inhibited the Gárdos effect induced by ATP depletion (fig.1B), or by propranolol (fig.1C). In another experiment (not shown), we found that the rate of the efflux of $^{86}Rb^+$ from pre-labelled red

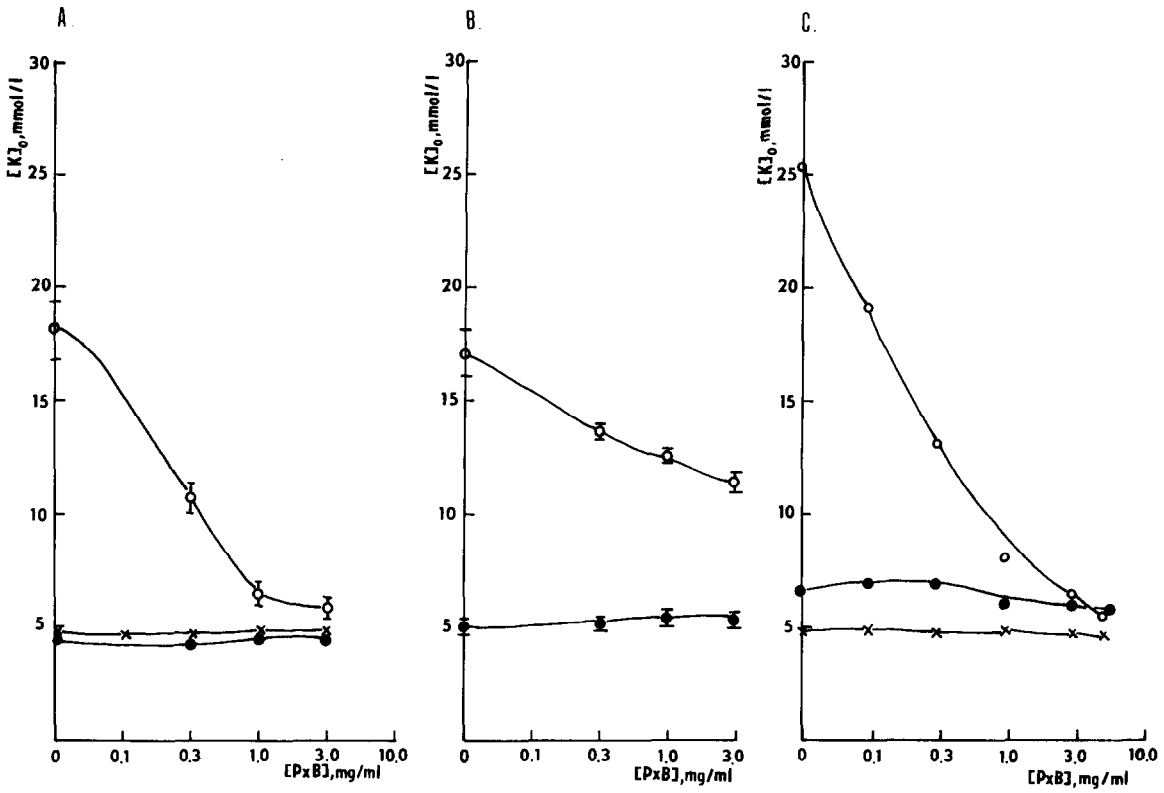

Fig.1. The inhibition by PXB of the (A) vanadate, (B) ATP-depletion-, or (C) propranolol-induced Gárdos effect. A dose-effect dependence. (A) Red cells were preincubated with (○,●) or without (×) 1 mM $NaVO_3$, and incubated with 2.5 mM Ca^{2+} (○,×) or with 2.5 mM EGTA (●) in the presence of indicated concentrations of PXB. Representative of 6 experiments. (B) ATP-depleted red cells were incubated with 2.5 mM Ca^{2+} (○) or with 2.5 mM EGTA (●) in the presence of indicated concentrations of PXB. Representative of 3 experiments. (C) Conditions as in A except that 0.5 mM propranolol was used instead of vanadate. Representative of 3 experiments. Where shown, the SE of duplicate measurements is indicated by a bar.

cells induced by A23187 plus Ca^{2+} was also slowed down by PXB. Thus, the conclusion could be made that the inhibition of the Gárdos effect by PXB is fairly independent of the mode of its induction.

The crucial step in inducing the Gárdos effect is the transfer of Ca^{2+} across the plasma membrane. Thus, the inhibition of the Gárdos effect by PXB could be explained by the inhibition of the Ca^{2+} influx. This possibility was tested using the vanadate-induced $^{45}Ca^{2+}$ uptake as a model (fig.2). PXB, in concentrations used for the inhibition of the Gárdos effect, did not inhibit the vanadate-induced $^{45}Ca^{2+}$ uptake and in several experiments even stimulated it. It should be mentioned at this point that propranolol induced the Gárdos effect without increasing significantly the intracellular Ca^{2+} concentration [2] (fig.2). In control experiments PXB at higher concentrations itself induced the uptake of $^{45}Ca^{2+}$. Its extent was much lower than that induced by vanadate. Propranolol slightly potentiated this effect (fig.2). Haemolysis also occurred at higher concentrations of PXB. The degree of the haemolysis was negligible as could be deduced from the control experiments shown in fig.1.

The efflux of K^+ during the Gárdos effect must be accompanied by the stoichiometrical efflux of Cl^- in order to maintain the electroneutrality. It has been shown that the transport of Cl^- is the rate-limiting step in the Gárdos effect [14]. Therefore the inhibition of the latter could result from the inhibition of the anion channel-mediated Cl^- efflux. We excluded this possibility by measuring the potential changes that occur during the Gárdos effect. The addition of Ca^{2+} to vanadate-treated red cells caused a hyperpolarization of the membrane (fig.3A). The addition of PXB prior to the addition of Ca^{2+} reduced the extent of hyperpolarization. No Ca-induced changes were ob-

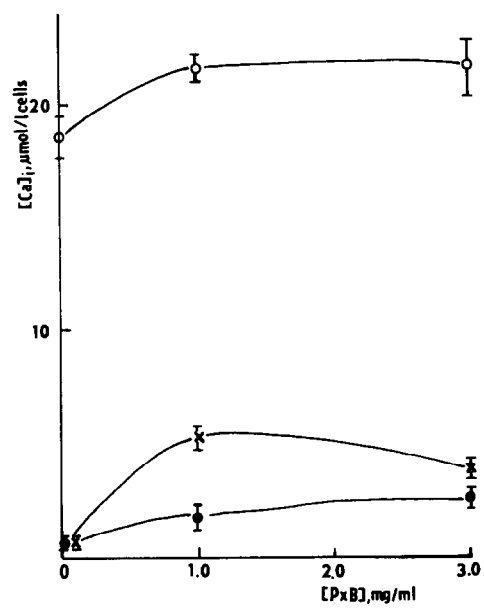

Fig.2. The effect of PXB on the $^{45}Ca^{2+}$ uptake observed in the presence of vanadate or propranolol. The $^{45}Ca^{2+}$ uptake was measured in the presence of 1 mM vanadate (○) or 0.5 mM propranolol (×), and indicated concentrations of PXB. Control cells (●) were incubated with the same volume of methanol (0.5%) as the vehicle for propranolol. Average values of parallel test tubes ± SD are shown. Typical of 11 (vanadate) or 3 (propranolol) experiments.

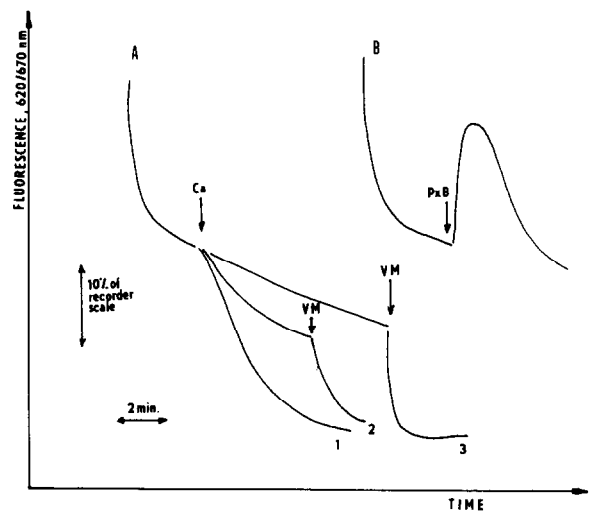

Fig.3. The inhibition by PXB of Ca^{2+}-induced hyperpolarization in vanadate-treated red cells. (A) Changes induced by 2.5 mM Ca^{2+} in the fluorescence of 3,3′-dipropylthiodicarbocyanine iodide in the red cell suspension in Na-rich medium were measured in vanadate-treated cells (traces 1,2) or in control cells (trace 3). VM denotes the addition of the K^+-specific ionophore valinomycin. PXB was added to trace 2 (1 mg/ml) prior to the addition of Ca^{2+}. The fluorescence traces were normalized to the same steady-state level of fluorescence prior to the addition of Ca^{2+}. (B) The fluorescence change induced by PXB (1 mg/ml). Representative of 2 experiments.

served in the control suspension. PXB itself induced a small increase of probe fluorescence (fig.3B) which was insensitive to the K^+ concentration in the medium, and could be ascribed to the displacement of the probe by PXB. The inhibition of the Ca^{2+}-induced membrane hyperpolarization by PXB in vanadate-treated cells means that PXB inhibits the moiety of the Ca^{2+}-activated K^+ channel rather than of the anion channel. In the latter case the acceleration of the hyperpolarizing response must have been observed.

The following experiments we performed in order to determine the role of protein kinase C in the inhibition of the Gárdos effect. We found that other inhibitors of protein kinase C, heparin (up to 30 U/ml) and quercetin [15] (up to 1 mM), did not inhibit the propranolol-induced Gárdos effect. Heparin was also ineffective in inhibiting the vanadate-induced Gárdos effect whereas quercetin was effective. This is, however, due to the direct chemical reaction between quercetin and vanadate which occurs even in the absence of red blood cells (not shown). In other experiments (not shown) we found that the phosphorylation pattern of membrane proteins and membrane-associated proteins did not change significantly upon the incubation of intact red cells pre-labelled with $^{32}P_i$, in the presence of Ca^{2+}, vanadate, PXB, or their combination.

4. DISCUSSION

The results show that the inhibition of the Gárdos effect in human red cells by PXB is due to the inhibition of the Ca^{2+}-activated K^+ channel. PXB as compared to previously known inhibitors of the Gárdos effect differs in its inhibitory mechanism. The important difference is the effect on the $^{45}Ca^{2+}$ uptake. Unlike quinidine [6], oligomycin (unpublished), divalent cations and HS-reagents [8], all of which inhibit the $^{45}Ca^{2+}$ uptake in parallel, PXB influences the $^{45}Ca^{2+}$ uptake in the opposite way (fig.2). In experiments which will be presented elsewhere we found that the $^{45}Ca^{2+}$ uptake preserved the features of a carrier-mediated transport in the presence of both vanadate and PXB. These results suggest that the activity of the Ca^{2+}-carrier is influenced by the activity of the Ca^{2+}-activated K^+ channel, and indicate that there may exist a dual mode of interaction between them. This may result from an intervention on either one, or both interacting components by the above mentioned inhibitors, and in intact red cells by a regulatory mechanism which is so far unknown. The attractive possibility that PXB affects the already mentioned transport systems via protein kinase C inhibition found only slight support from our experiments so far, although protein kinase C activity in human red cells has recently been demonstrated [16]. However, the possibility is still open that a soluble regulatory protein is phosphorylated by protein kinase C which was lost during a procedure for the preparation of samples for the electrophoresis, and which could be a target for the action of PXB. The involvement of a soluble protein for the expression of the Ca^{2+}-activated K^+ channel has indeed recently been demonstrated [17].

The involvement of protein kinase C in the activation of the Gárdos effect could explain the fact that propranolol induces the Gárdos effect without a significant increase of intracellular Ca^{2+} concentration [2] (fig.2) if one takes into account that protein kinase increases the affinity for Ca^{2+} of some of its target processes [18,19].

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